Mushroom Emergence Detected by Combining Spore Trapping with Molecular Techniques

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Keywords: *Propagules, Ectomycorrhizal, Saprotroph, DNA barcoding, molecular identification, dispersion, fungi, Lactarius*

ABSTRACT

Obtaining reliable and representative mushroom production data requires time-consuming sampling schemes. In this paper, we assessed a simple methodology to detect mushroom emergence by trapping the fungal spores of the fruiting body community in plots where mushroom production was determined weekly. We compared the performance of filter paper traps with that of funnel traps and combined these spore trapping methods with species-specific quantitative real-time PCR and Illumina MiSeq to determine the spore abundance. Significantly more MiSeq proportional reads were generated for both ectomycorrhizal and saprotrophic fungal species using filter traps than were obtained using funnel traps. The spores of 37 fungal species that produced fruiting bodies in the study plots were identified. Spore community composition changed considerably over time due to the emergence of ephemeral fruiting bodies and rapid spore deposition (lasting from one to two weeks), which occurred in the absence of rainfall events. For many species, the emergence of epigeous fruiting bodies was followed by a peak in the relative abundance of their airborne spores. There were no relationships between fruiting body yields and spore abundance in time for only two out of seven fungal species. There was no relationship between fruiting body yields and their spore abundance at plot level, indicating that some of the spores captured in each plot were arriving from the surrounding areas. Differences in fungal detection capacity by spore trapping might indicate different dispersal ability between fungal species. Further research could help to identify the spore rain patterns for most common fungal species.

IMPORTANCE

Mushroom monitoring represents a serious challenge in economic and logistical terms because sampling approaches demand extensive field work at both the spatial and temporal scales. In addition, the identification of fungal taxa depends on the expertise of experienced fungal taxonomists. Similarly, the study of fungal dispersal has been constrained by technological limitations, especially because the morphological identification of spores is a challenging and time-consuming task. Here, we demonstrate that spores from ectomycorrhizal and saprotrophic fungal species can be identified using simple spore traps together with either MiSeq fungal-specific amplicon sequencing or species-specific quantitative real-time PCR. In addition, the proposed methodology can be used to characterize the airborne fungal community and to detect mushroom emergence in forest ecosystems.

INTRODUCTION

Wild edible fungi are highly important non-wood forest products and are increasingly in demand at food markets worldwide (1). Up to 268 fungal taxa have been authorized to be commercialized in Europe (2), of which the most important marketed mushrooms are *Boletus edulis*, *Cantharellus cibarius*, *Lactarius deliciosus*, *Morchella esculenta*, and *Agaricus campestris* (2, 3). Spain, the Netherlands, France, and Poland are the largest mushroom producers in Europe (2). Despite the increasing interest and importance of mushrooms as non-wood forest products, mushroom production has only recently been included as a target in forest management and planning alongside the traditional goal of wood production (4). The inclusion of mushroom production into forest management planning requires the ability to evaluate potential mushroom yields in quantifiable terms. However, the variability in mushroom biomass across years, the ephemeral

cycle of several fruiting bodies that are only observable for a few days, and the time needed to sample representative mushroom populations (5) represent serious challenges for monitoring purposes (6). In this context, new techniques that do not require the collection of mushrooms could improve the estimation of mushroom yields. Except for a few cases (7), no correlations have been found between the belowground mycelial biomass of specific species and their mushroom production (8–10), even though positive correlations have been found between root mycorrhizal colonization and soil mycelia (11). In this regard, new monitoring approaches using fungal spores, such as spore trapping, could represent a new way of addressing this challenge. Spore detection could be used to evaluate the fruiting bodies present without the need for regular or repeated samplings over time.

Traditionally, spore trapping has been used to detect and monitor airborne spores, particularly those of fungal pathogens (12). However, recently, spore trap methods have been combined with molecular techniques such as quantitative real-time PCR (qPCR) (13, 14). Spore traps need to be simple, robust devices for effective use in forest stands; they also need to be easy to handle and have low maintenance costs (14, 15). Active traps (e.g., volumetric or cyclonic traps) trap fungal spores more effectively than passive traps (12); however, they have some disadvantages when used under forest conditions, for example, they require electric power. By contrast, passive traps, such as filter or funnel traps, can be placed anywhere (13). Previous studies have shown that passive traps can capture ectomycorrhizal fungal propagules. These passive traps consisted of simple funnels attached to jars (16, 17). However, even simpler spore traps have been used to capture pathogenic fungi, such as filter traps (13). Although these traps collect spores passively, funnel-based traps collect spores in a jar, whereas filter traps retain spores within the filter, which

may mean that the spore collection performance of these two traps is different. Despite the potential of these traps to capture fungal spores, to date, no quantitative relationship between fruiting bodies and airborne spores has been established.

Besides the type of trap, the dispersal capacity of the spores also affects spore detection. Unlike highly sporulating fungi, such as puffballs or several mold or yeast species that dominate the airborne community (18), ectomycorrhizal basidiomycetes seem to disperse spores less abundantly (19–21). Problems of detecting rare species can be circumvented by the use of molecular techniques such as qPCR (22), which can detect even small amounts of fungal DNA, although specific primers need to be designed for the target species (13). High-throughput DNA sequencing technologies enable the relative proportions of each of the identified operational taxonomic units (OTUs) to be determined in a given sample (23). Besides monitoring mushroom emergence, this approach could also be used to understand fungal dispersal. To sum up, spore traps combined with qPCR could be used to detect airborne fungal spores of specific species, while spore traps in combination with high-throughput sequencing approaches could be used as a generic tool to detect and identify airborne spores of different taxa.

We hypothesized that: (i) funnel and filter traps in combination with qPCR or MiSeq would differ in their capacity to detect spores; (ii) there is a relationship between fruiting body yields and spore abundance over time (temporal relationship) and over plots (spatial relationship). To test these hypotheses, we identified the relative abundance of the spores by trapping spores during an 8-week period in the fall of 2014 in a *Pinus pinaster* forest in NE Spain. In parallel, we also determined the taxonomic identity and the yields of the fruiting body community.

First, we studied *Lactarius vinosus* as a model species to compare the two molecular techniques (qPCR vs. MiSeq). We chose this species because of its commercial interest. It belongs to the *Lactarius* group *deliciosus*, which has well-established markets across Europe, Asia, and North Africa (1). Furthermore, this species is highly appreciated both by mushroom pickers and consumers in Spain (24, 25). The commercialization of *Lactarius* group *deliciosus* has led to the sale of almost 500,000 kg of *L. vinosus* a year at the three most important Spanish markets; the estimated market value is 5.3 M € $year^{-1}$ (25).

Second, we studied the relationship between the spore abundance of a species and the yield of fruiting bodies from a temporal (week) and spatial (plot) perspective. For this, we compared the abundance of the fruiting bodies of the 12 most abundant fungal species (6 ectomycorrhizal and 6 saprotrophic fungal species) found in the fruiting body community.

RESULTS

Fungal species producing fruiting bodies in the studied plots

Among the sampled fruiting bodies, we found 71 fungal species belonging to the ectomycorrhizal or saprotrophic fungal guilds. Among these ectomycorrhizal and saprotrophic species, we identified 37 species that were also present in the spore community. Of those species, 18 species produced fruiting bodies in at least two plots for a two-week period, and the 12 most abundant species (6 ectomycorrhizal and 6 saprotrophic fungal species) were selected for analysis (Table 1). Thirteen species producing fruiting bodies did not have an internal transcribed spacer (ITS) reference in the UNITE (Unified system for the DNA based fungal species linked to

120 classification) database or in the INSD (International Nucleotide Sequence Database) and,
121 therefore, these species were not searched for within the spore community.

122 Spore abundance estimated by trapping techniques and molecular methods

123 MiSeq data revealed that the relative abundance of *L. vinosus* was significantly higher in filter
124 traps than in funnel traps (F-value$_{[1,15]}$ = 4.982, P<0.001, Table 2), representing on average 5.55
125 and 0.48 per thousand of the total number of reads, respectively (i.e., nearly 10-times more
126 abundant in filter traps than in funnel traps; Table 2). However, when using qPCR, the number of
127 *L. vinosus* spores trapped by the filter and funnel traps did not seem to be significantly different
128 (F-value$_{[1,15]}$ = 0.062, P = 0.951; Table 2). When using funnel trap data, the spore abundance of *L.*
129 *vinosus* estimated by qPCR correlated with that estimated by MiSeq (F-value$_{[1,59]}$ = 27.53,
130 P<0.001, R^2 = 0.3, Fig. 1a). However, there was not relationship between spore abundance of *L.*
131 *vinosus* detected at the filter traps with the abundance detected at the funnel traps (F-value$_{[1,14]}$ =
132 0.92, P = 0.352, Fig. 1b)

133 The higher relative abundance of *L. vinosus* spores detected by MiSeq when using filter traps
134 rather than funnel traps was also observed for most of the saprotrophic and ectomycorrhizal
135 fungal species studied (F-value$_{[1,15]}$ = 5.712, P<0.001, Table 2). When comparing guilds, we
136 found that selected yeast species were better represented in funnel traps than in filter traps (F-
137 value$_{[1,15]}$ = 4.703, P<0.001, Table 2), but no differences between spore trap types were observed
138 for mold species.

139 Weekly relationship between fungal spores and the production of fruiting bodies

140 The spore community composition varied significantly across the weeks ($F_{[7,59]} = 3.727$, $R^2 =$
141 0.27, $P<0.001$) and plots ($F_{[7,59]} = 3.458$, $R^2 = 0.25$, $P<0.001$). The temporal effect (across weeks)
142 was as high as the spatial effect (across plots), and together accounted for more than 50% of the
143 total variation. The abundance of spores of different species found in the traps fluctuated over the
144 sampling period, especially those of ectomycorrhizal fungal species (Fig. 2a, 2b).

145 Three saprotrophic (*Marasmius androsaceus*, *Clitocybe phaeophthalma*, and *Hypholoma*
146 *fasciculare*) and two ectomycorrhizal fungal species (*Russula torulosa* and *Russula chloroides*)
147 showed signs of having highly ephemeral fruiting bodies. Spore production by most of these
148 species peaked one or two weeks after fruiting body production but then ended abruptly (Fig. 3a,
149 3b). Species with at least three spore production peaks during the study period showed a
150 significant relationship between fruiting body yields and their spore abundance; for example,
151 *Mycena pura* (F-value$_{[1,51]} = 36.97$, $P<0.001$, $R^2 = 0.96$), *Lactarius deliciosus* (F-value$_{[1,51]} =$
152 11.56, $P = 0.001$, $R^2 = 0.68$), *Lactarius vinosus* (F-value$_{[1,51]} = 57.39$, $P<0.001$, $R^2 = 0.77$),
153 *Leucopaxillus gentianeus* (F-value$_{[1,51]} = 12.58$, $P<0.001$, $R^2 = 0.59$), and *Tricholoma terreum* (F-
154 value$_{[1,51]} = 20.89$, $P<0.001$, $R^2 = 0.58$). This relationship was also confirmed for *L. vinosus* using
155 qPCR data (F-value$_{[1,48]} = 29.24$, $P<0.001$, $R^2 = 0.79$). *L. vinosus* spore abundance patterns
156 detected using either Illumina MiSeq or qPCR data were very similar and both techniques
157 showed that the production of *L. vinosus* spores peaked between October 20th and October 29th.
158 This peak was estimated to represent an average of 1.5×10^6 *L. vinosus* spores $\times$ trap^{-1} (Fig. 4).

159 In contrast to the species already mentioned, *Lycoperdon perlatum* (F-value$_{[1,51]} = 0.01$, $P =$
160 0.917) and *Inocybe glabripes* (F-value$_{[1,51]} = 0.89$, $P = 0.349$) did not display a significant
161 relationship between fruiting body yield and spore abundance. For example, the spore abundance

of *L. perlatum* increased with time and did not correspond to the variable fruiting body production (Fig. 3a), and the spore abundance of *I. glabripes* peaked at the start of the season, which was not reflected at the fruiting body level (Fig. 3b).

Relationship between spores and fruiting body production at plot level

At plot level, only spores of saprotrophic fungal species correlated with fruiting body production ($F_{[1,48]} = 4.11$, $P = 0.048$, Fig. 5a). These significant differences were mainly attributed to the effect of one plot, in which large quantities of both spores and fruiting bodies were recorded. Nevertheless, no relationship was observed between spores of ectomycorrhizal fungi and their fruiting body yields ($F\text{-value}_{[1,48]} = 0.41$ $P = 0.527$, Fig. 5b). Only five species were found in more than two plots: *Inocybe glabripes*, *Lactarius deliciosus*, *Lactarius vinosus*, *Lycoperdon perlatum*, and *Mycena pura*. None of these species showed a significant relationship at plot level between spores and their fruiting bodies ($P>0.05$). Thus, in most cases, fruiting bodies were found in only a few plots but their spores were collected in a greater number of plots (e.g., *Mycena pura,* Fig. 5c). In other cases, the plots with high levels of fruiting body production also had the highest spore abundance, but no quantitative relationships were found (e.g., *Lactarius vinosus*, Fig 5d). Amongst the saprotrophic fungal species there was a clear altitudinal pattern ($F_{[1,6]} = 2.48$, $P = 0.047$, Fig. 6), with both fruiting bodies and spores decreasing towards higher altitudes, whereas no clear altitudinal pattern was observed amongst ectomycorrhizal fungal spores ($F_{[1,6]} = -0.28$, $P = 0.785$, Fig. 6).

DISCUSSION

In this study, we showed that spore trapping coupled with qPCR or MiSeq can detect the emergence of epigeous mushrooms. This study demonstrates that qPCR and MiSeq can be used to detect fungal emergence. In general, a one-week delay between mushroom emergence and airborne propagule detection was found using spore traps. This delay was most likely caused by a combination of i) the time needed for mushrooms to mature and produce spores, and ii) the time needed for spore ejection and deposition in the spore traps. However, an alternative explanation for the observed delay might be that our fruiting body sampling scheme likely favors the sampling of immature fruiting bodies before they initiate sporulation. It is generally accepted that spore deposition is driven by meteorological conditions. In theory, the initial phase of spore dispersal of some basidiomycete mushroom-forming species involves the discharge of basidiospores from the gill by a mechanism promoted by a droplet, also known as a Buller's drop (26), which is normally stimulated by the secretion of mannitol and other sugars (27). Then, convective airflows promoted by the pileus enhance the vertical movement of these spores, which may then reach dispersive winds (28). Finally, these spores may fall by gravity or rainfall events. However, in our study we observed that spore deposition occurred regardless of rainfall events, indicating that spore deposition is not necessarily driven by meteorological conditions such as precipitation events (29). Future studies should focus on gaining a better understanding of the potential delay in spore detection and the factors driving spore deposition, such as the effect of winds on the movement of these spores.

In this study, we observed an interspecific short temporal variation in the abundance of spores, especially those produced by ectomycorrhizal fungal species. This finding agrees with the literature because spores can remain airborne for between a day and up to a few weeks,

204 depending on their size and aerodynamic properties (30). The temporal variation in spore
205 abundance has been reported previously (16, 20), and this variation has been attributed to
206 different abiotic parameters such as wind direction, rainfall, and species-specific life-history traits
207 (29,31-32). Based on our results, spore detection was related to mushroom production peaks, thus
208 it seems likely that seasonal differences in the composition of the airborne fungal community
209 could also be partly driven by mushroom phenology (20, 33).

210 Despite the observed relationship between mushroom yields and spore abundance across the
211 sampling weeks, there was not relationship at the spatial scale where mushroom production was
212 assessed i.e. plots of 10 × 10 m. Spores were captured in plots without fruiting bodies of the
213 corresponding species, indicating that these spores were produced outside the sampled plots. The
214 spores of ectomycorrhizal fungal species are usually dispersed approximately 10 to 1000 meters
215 (16, 19, 21) depending on the species (17). The large amount of spores produced per fruiting
216 body, e.g., $1.1 \times 10^8 – 1 \times 10^{10}$ spores (34, 35), makes it possible for spores of these species to be
217 dispersed long distances, particularly if carried away by turbulences. The fine mapping of spore
218 captures in future studies could be used to indirectly estimate dispersal curves for several species
219 and therefore understand better how these fungal species disperse.

220 MiSeq analysis detected significantly greater relative proportions of our 12 selected fungal
221 species belonging to the saprotrophic or ectomycorrhizal guilds in filter traps than in funnel traps.
222 Funnel traps trapped a greater number of species from other fungal guilds such as yeasts than the
223 filter traps, which translated into a relative decrease in the proportion of saprotrophic and
224 ectomycorrhizal MiSeq reads. Differences between samples could arise because of the way in
225 which samples were handled. Spores captured at the funnel traps were first filtered *in situ* and

226 subsequently collected inside a jar with water (17), which could favor the growth of some
227 opportunistic fungi such as yeasts. Both fungal-specific amplicon sequencing and species-specific
228 quantitative real-time PCR successfully detected mushroom production peaks. Both methods
229 revealed an almost identical pattern of spore abundance across the weeks for *L. vinosus*. The use
230 of MiSeq avoids the need to design specific primers for each target species. However, the use of
231 high-throughput sequencing data as a semi-quantitative measure has been the subject of
232 discussion, mostly because of the variation in ribosomal gene copy numbers among different
233 fungal species (36), the read length biases caused by current high-throughput sequencing
234 platforms (37), the DNA extraction efficiency (38), or biases due to interspecific primer binding
235 differences. Despite these limitations, read abundance may still be used as a semiquantitative
236 measure of abundance (relative proportions) (23).

237 We showed that a peak in ephemeral fruiting bodies was followed by a peak in the numbers of
238 spores that were trapped. For most of the species, the spore peak mostly disappeared two weeks
239 after mushroom emergence reached its highest level. Nevertheless, a quantitative relationship
240 between fruiting body yields and their fungal spores was not observed for *Inocybe glabripes* and
241 *Lycoperdon perlatum*. Although the spore abundance and fruiting body yields observed for *I.*
242 *glabripes* showed a similar pattern, the lack of relationship between these two measures was most
243 likely caused by a peak in spore abundance that was not reflected at the fruiting body level.
244 Interestingly, the spore abundance pattern for *L. perlatum* suggests a progressive and slow spore
245 deposition over time; the lack of relationship between spore abundance and time could be
246 because these spores are rarely falling unless a rainfall event occurs, such as the event occurred
247 during the week of December 4th, which most likely washed away all the spores.

248 Some species produced fruiting bodies but their spores were not found among the spore
249 community. Possible explanations for this include: i) these species had a very short range of
250 dispersal, or ii) their sporulation was very small compared with the rest of the species.
251 Alternatively, these species could have a very long ITS2 sequence and, thus, be underrepresented
252 using MiSeq sequencing. We investigated whether this was a plausible explanation by studying
253 the ITS2 region length of these species using ITS sequences from UNITE, specifically if the
254 region theoretically amplified by the primers was longer and, if the DNA sequence where each
255 primers binds was different for the fungal species not detected at the spore traps. However, the
256 length of the ITS2 region of these species was similar to that of other species that were
257 successfully captured by the spore traps. Primer sequence mismatch was also discarded as an
258 alternative explanation because these species did not show any sequence mismatch where the
259 primer binds. Thus, discarded these last possibilities, and given that fruiting bodies were
260 collected <10 m from spore traps, we hypothesize that low sporulation might be the reason for
261 the lack of detection of these species among the spore community.

262 Filter traps are more suitable for high-throughput sequencing approaches targeting saprotrophic
263 or ectomycorrhizal fungal species. Our method represents a potential improvement on mushroom
264 sampling approaches, as current sampling schemes are highly time-consuming and require expert
265 knowledge to identify the fungal taxa (5). Here, we showed that mushroom production and fungal
266 diversity of fungi with epigeous fruiting body structures can be monitored accurately by spore
267 traps. Thus, there are less time-consuming alternatives for monitoring mushrooms than sampling
268 fruiting bodies, which are fundamental for an effective management planning of non-wood forest
269 products.

MATERIALS AND METHODS

Study area

The study was carried out at a long-term experimental set-up located in the Natural Area of Poblet (Northeast Spain, 41° 21' 6.4728" latitude N and 1° 2' 25.7496" longitude E), which is characterized by a Mediterranean climate, with an average annual temperature of 11.8°C and annual rainfall of 665.5 mm. The long-term experimental site comprises 28 fenced plots, of which we selected 8 plots (10 × 10 m), where fruiting bodies have been continuously monitored every fall since 2008 (39). Even-aged (60-years-old) *Pinus pinaster* and *Quercus ilex* are the co-dominant species growing in the plots. Additional information about the study area and the plots can be found in Table 3.

Fruiting body sampling

All plots were sampled for mushroom production weekly during the fall (October–December 2014) following previously described methods (5). In short, all the fruiting bodies within the plot and with a minimum cap size of 1 cm were sampled on Thursdays to minimize errors resulting from recreational weekend collectors picking mushrooms. The plots were chosen according to the productivity gradient for *Lactarius vinosus* (from the highest to the lowest production), which was based on continuous production data, which have been recorded since 2008 (39). All the fruiting bodies from all the species found within the plots were brought to the laboratory the same day, identified, cleaned, and counted. Fruiting bodies were dried in an air-vented oven at 35–40°C, and weighed to the nearest 0.01 g. Data in this study were expressed as the number of

fruiting bodies (*n*) per plot in kg × ha^{-1} to show the temporal variation of *L. vinosus* fruiting bodies over time.

Spore trapping

Two types of spore traps were installed: filter (13) and funnel traps (16). Both traps were placed one meter apart in the center of each plot and captured spores 30 cm above ground level.

Funnel traps consisted of 15-cm-diameter funnels attached to 1-liter dark jars, with a 50-µm nylon mesh fixed at the bottom of the funnel. These traps were erected a week after the first mushroom of *L. vinosus* was observed (October 14th, 2014) and remained in place until December 11th, 2014, when no *L. vinosus* fruiting bodies were observed. Each week the funnels were rinsed with ultrapure water (MilliQ) to collect any spores and then the jars were immediately removed and replaced with sterile jars. Liquids from the traps were filtered using sterile filter papers (90-mm diameter: Whatman no. 1) within 48 hours of collection. Sample filtering was conducted in a flow chamber to prevent potential contamination. Filters were stored at –20ºC until further analysis.

Filter traps consisted of a filter paper (Whatman no. 1, 90 mm diameter filter paper) placed over a metal mesh, supported by a metal clamp and attached to a vertical support 30 cm above ground level. The filter papers in the filter traps were sampled simultaneously to the funnel traps during the two most productive weeks when *L. vinosus* fruiting body production was greatest (from October 14th until October 29th, 2014), and stored at –20ºC after sampling.

Spore trap sample processing and DNA extraction

Filters containing spores from the filter and the funnel traps were cut in half and placed in separate 50-ml falcon tubes. A solution of 20-ml sodium dodecyl sulfate (SDS buffer) was added to each tube before incubating at 65°C for 90 minutes. The tubes were vortexed three times before removing the filter from each tube. Twenty milliliters of 2-propanol were added to the resulting solution and then left overnight at room temperature. After centrifugation (700 × *g* for 10 min) the supernatant was carefully removed and 700 µl of SL2 lysis buffer (NucleoSpin® NSP soil DNA extraction kit, Macherey-Nagel, Duren, Germany) was added. The resultant solution was vortexed and transferred to a 2-ml tube. After the addition of SX Enhancer (NucleoSpin® NSP soil DNA extraction kit), the spore solution was homogenized in a FastPrep®-24 system (MP Biomedicals) at 5,000 × *g* for 30 s (twice) following the instructions provided by the manufacturer. The DNA obtained was eluted in a 50-µl elution buffer.

Quantification of *Lactarius vinosus* using Real-Time PCR

For *Lactarius vinosus* spore quantification, we used a species-specific hydrolysis probe (40). In order to obtain comparable results between different plates, we prepared standard curves using known amounts of *L. vinosus* DNA, which were extracted with the NucleoSpin® NSP soil kit (Macherey-Nagel, Duren, Germany). C_T values were converted to the number of *L. vinosus* spores in each reaction using serial DNA dilutions of known amounts of spores, starting with 15.10×10^6 spores and ending with 151.2 spores. Results are expressed as the number of spores × trap sample $^{-1}$.

Real-time PCR reactions were prepared using 2X Premix Ex Taq™ (Takara Bio Europe SAS, France) following the manufacturer's instructions. The reaction mix was prepared as follows: 5 µl of DNA template, 400 nM of each oligonucleotide, 200 nM of TaqMan probe, 0.8 µl of ROX, and a volume of water (HPLC) to adjust the final reaction volume to 20 µl. The cycling conditions in the StepOnePlus instrument (Applied Biosystems) were as follows: 30 s at 95°C, followed by 40 cycles at 95°C for 5 s and at 60°C for 34 s. There were three replicates of each sample and the standards were included in the analysis, as well as a negative control using HPLC water instead of the template. Only amplification efficiencies ranging from 95% to 105% were accepted and considered for analysis. qPCR reactions, processing of data, and quantification of *L. vinosus* DNA were performed following the methods described in a previous study (11).

Spore trap sample sequencing using Illumina MiSeq

Each spore trap sample was PCR-amplified using the primers fITS7 and ITS4 (41), which amplify the ITS2 region of the rDNA. Both primers were tagged using unique DNA sequences composed of eight bases. A PCR cycle test was performed prior to randomly selected samples in order to perform the minimum number of PCR cycles possible. PCR amplifications of samples and both negative controls from DNA extraction and PCR were conducted in a 2720 Thermal Cycler (Life Technologies) in 50 µl volumes. The final concentrations in the PCR reaction mixture were: 25 ng of template, 200 µM of each nucleotide, 2.75 mM $MgCl_2$, 200 nM of each primer, 0.025 U μl^{-1} polymerase (DreamTaq Green, Thermo Scientific, Waltham, MA) in 1× buffer PCR. The cycling conditions for PCR were: 5 min at 95°C, followed by 24–30 cycles of 30 s at 95°C, 30 s at 56°C, and 30 s at 72°C, and a final extension step at 72°C for 7 min before storage at 4°C. Each sample was amplified in triplicate, purified using an AMPure kit (Beckman

Coulter Inc. Brea, CA), and quantified using a Qubit fluorometer (Life Technologies, Carlsbad, CA). Equal amounts of DNA from each sample were pooled before sequencing. The final equimolar mix was purified using an EZNA Cycle Pure kit (Omega Bio-Tek). Quality control of purified amplicons was carried out using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA) 7500 DNA chip. Libraries were prepared from ~10 ng of fragmented DNA using the ThruPLEX-FD Prep kit. The samples were sequenced using the Illumina MiSeq platform, with 300-bp paired-end read lengths, generating 13.4 million sequences.

Quality control and bioinformatic analysis

Quality control, filtering, and clustering were assessed using the SCATA pipeline (scata.mykopat.slu.se). Sequences were filtered to remove data with a minimum allowed base quality score of <10 at any position, an average quality score of <20, and a minimum sequence length of 200 bp, using the amplicon quality option. Sequences were also screened for primers (using 0.9 as a minimum proportional primer match for both primers) and sample tags. We used 'usearch' as a search engine, considering a minimum match length of 85%. Homopolymers were collapsed to 3 bp before cluster analysis. Pairwise alignments were conducted using a mismatch penalty assigned of 1, a gap open penalty of 0, and a gap extension penalty with a value of 1. Sequences were clustered in OTUs with single linkage clustering, using 1.5% as a threshold distance with the closest neighbor. After quality control and clustering, all tags were identified and tag jumps were removed from the database (42). A tag jump has been defined as the generation of artifactual sequences in which amplicons carry different tags to those originally applied. The raw sequence reads have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA352156.

Identification of the fungal clusters

From a total of 13,408,476 MiSeq DNA sequences, 1,992,529 passed the quality control. Of these, 95,104 (4.77%) sequences were discarded because they had two different tags. Finally, 1,897,425 reads were obtained, with an average of 19,361 reads per sample.

We searched among the most abundant 2,400 OTUs (representing OTUs with more than 20 read counts) for those potential fungal species representing the fruiting body community. We first identified the OTUs using the seriateBLAST option in the UNITE database (43). We included DNA sequences from both UNITE and INSD, but preference was given to the UNITE reference for identification. In case we could not find any given species, we verified that each species had a known reference ITS sequence by checking UNITE and INSD, in order to differentiate those species not found among the spore community from those without a known ITS reference sequence. Taxonomic assignment of the OTUs was given using a 98.5% sequence similarity threshold, and all the species found at or above this threshold were included in each OTU. Read count data were transformed to relative proportions and data are shown as counts per thousand of the total read numbers per sample.

Statistics

All statistical analyses were implemented in the R software environment (version 2.15.3; R Development Core Team 2013) using the "nlme" package for linear mixed models (LME (44)), the vegan package (45) for the multivariate analysis, and the "gstat" (47) and "maps" (48) packages for spatial kriging.

Applied and Environmental Microbiology

Comparison of spore traps and of the two molecular methods used to quantify the fungal spores

LME models were used to test differences between spore traps (filter spore traps and funnel traps) using both qPCR and MiSeq data for *L. vinosus* and only MiSeq data for the other species. In these models, the temporal dependency was considered by defining plots nested with 'week' as a random factor, whereas the square root spore abundance of 12 each species was defined as a fixed factor. In these analyses, we included the 12 species considered in this study (6 ectomycorrhizal and 6 saprotrophic fungal species) in separate models and randomly selected yeast (i.e., *Cryptococcus* spp. and *Rhodotorula* spp.) and mold species (*Mortierella elongata* and *Trichoderma* sp.) to test whether the hypothetical effect of the spore trap type was due to the increase of some of these fungal guilds.

Temporal relationship between fungal spores and mushroom yields

Temporal (week) and spatial (plot) effects on community data were tested using permutational multivariate analysis of variance (PERMANOVA) based on distance matrices (using the function adonis and Bray–Curtis distances). Plot and week were included as factors, and community data were previously Hellinger transformed. For this analysis, the fruiting body community data represented species that produced fruiting bodies in at least two plots for at least two weeks.

We analyzed the temporal (week) linear relations between spore abundance and the mushroom yields using LME models, using square root transformed qPCR and MiSeq data. To test for lagged relationships, different LME models were tested, with and without accounting for one-week temporal autocorrelation among observations (corAR1(form~week)). The best model fit

was selected using the Akaike information criterion (AIC) value. We confirmed this lagged relationship with LME models using data in which spores were 1-week manually lagged. Only species that produced fruiting bodies for at least two weeks were considered for analysis. As mushroom fresh weight, dry weight, and the number of mushrooms ha^{-1} were highly correlated ($P<0.001$; $R^2 = 0.9$), we only used fruiting body counts for analysis. Data were modeled with plots defined as random to deal with the intra-plot variation and either qPCR data (*L. vinosus*) or MiSeq data (for each species) were used as explanatory variables.

Relationship between fungal spores and mushroom yields at plot level

The distribution of fungal spores and mushroom counts at the spatial scale was obtained by ordinary kriging interpolating each value using the inverse distance weighting (IDW) function. Interpolated values were graphically represented using Universal Transverse Mercator (UTM) coordinates (European Datum 1950, ED50). The relationship between spores and fruiting bodies over the plots was tested with LME models, using only species found in more than two plots. In this case, data were modeled with week defined as random to deal with the temporal variation.

ACKNOWLEDGMENTS

The authors are very grateful to the PNIN of Poblet for supporting the process of installing and maintaining the experimental plots. We also thank Katarina Ihrmark and Johanna Boberg for providing the protocol and for advice on how to process and analyze the spore trap samples. We thank Josep Miró and Francesc Bolaño for their assistance with collecting the spore trap and fruiting body samples. We thank Caroline Woods for linguistic revision and for helping to clarify the manuscript.

FUNDING INFORMATION

This work was supported by an STSM Grant from COST Action FP1203 and by the Spanish Ministry of Economy and Competitiveness (MINECO) through the projects MICOGEST AGL2012-40035-C03 and MYCOSYSTEMS AGL2015-66001-C3. Carles Castaño received the support of the Secretaria d'Universitats i Recerca del Departament d'Economia i Coneixement de la Generalitat de Catalunya through the program of Doctorats Industrials. José Antonio Bonet is supported by the Serra-Hunter fellowship and Josu G. Alday is supported by the Juan de la Cierva fellowship (IJCI-2014-21393). The funders had no role in the study design, data collection, and interpretation, or in the decision to submit the work for publication.

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568 project.org/web/packages/maps/index.html).

569

570 TABLE 1. Selected most abundant species (species found at least twice in two different plots). Identification was based on comparison

571 with ITS2 reference sequences deposited in UNITE. Ecology refers to the functional guild of the species (ectomycorrhizal or saprotrophic).

572 Frequency refers to the order of frequency in terms of the total number of MiSeq reads among the whole propagule community.

Potential species	Ecology	Reference	Species Hypothesis Concept	DOI	Frequency
Inocybe glabripes Ricken	Ectomycorrhizal	UDB000099	*Inocybe glabripes*	DOI: 10.15156/BIO/SH174191.07FU	1620
Lactarius deliciosus (L.) S. F. Gray	Ectomycorrhizal	UDB011514	*Lactarius deliciosus*	DOI: 10.15156/BIO/SH220107.07FU	227
Lactarius vinosus Quél	Ectomycorrhizal	UDB000876	*Lactarius semisanguifluus*	DOI: 10.15156/BIO/SH220111.07FU	104
Russula chloroides (Kromb.) Bres.	Ectomycorrhizal	FM999633	*Russula chloroides*	DOI: 10.15156/BIO/SH220523.07FU	260
Tricholoma terreum (Sch.) Kumm.	Ectomycorrhizal	UDB015079	*Tricholoma gausapatum*	DOI: 10.15156/BIO/SH219345.07FU	168
Russula torulosa Bres.	Ectomycorrhizal	UDB011110	*Russula torulosa*	DOI: 10.15156/BIO/SH186211.07FU	877
Clitocybe phaeophthalma (Pers.) Kuyper	Saprotrophic	JX514120	*Singerocybe umbilicata*	DOI: 10.15156/BIO/SH191019.07FU	1709
Hypholoma fasciculare (Huds.) Kumm.	Saprotrophic	KF373785	*Hypholoma fasciculare*	DOI: 10.15156/BIO/SH201439.07FU	32
Leucopaxillus gentianeus (Quél.) Kotl.	Saprotrophic	UDB011628	*Leucopaxillus gentianeus*	DOI: 10.15156/BIO/SH030478.07FU	50
Marasmius androsaceus (L.) Fr.	Saprotrophic	AF519893	*Gymnopus androsaceus*	DOI: 10.15156/BIO/SH183805.07FU	1680
Mycena pura (Pers.) Kumm.	Saprotrophic	JF908417	*Mycena diosma*	DOI: 10.15156/BIO/SH186350.07FU	79
Lycoperdon perlatum Pers.	Saprotrophic	UDB023596	*Lycoperdon perlatum*	DOI: 10.15156/BIO/SH175885.07FU	51

573

574 TABLE 2: Differences in the relative proportions of ectomycorrhizal and saprotrophic species detected by filter traps vs. funnel traps. A
575 few of the most abundant yeast and mold species are also included in the analysis to determine whether the relative proportion of
576 ectomycorrhizal and saprotrophic species detected by funnel traps was smaller than that detected by filter traps owing to the trapping of a
577 larger proportion of yeast or mold species by funnel traps.

		Mean proportions		Effects				
Ecology	Species	Filter trap	Funnel trap	Value	SE	df	*t*-value	*P*-value
Ectomycorrhizal	*Russula torulosa*	0.354	0.003	–0.262	0.126	15	–2.082	0.055
Ectomycorrhizal	*Inocybe glabripes*	0.079	0.015	–0.089	0.07	15	–1.267	0.225
Ectomycorrhizal	*Lactarius deliciosus*	2.408	0.075	–1.09	0.228	15	–4.793	**<0.001**
Ectomycorrhizal	*Lactarius vinosus* (MiSeq)	5.551	0.450	–1.531	0.307	15	–4.982	**<0.001**
	Lactarius vinosus (qPCR)	2.260	2.676	–0.016	0.268	15	–0.062	0.951
Ectomycorrhizal	*Russula chloroides*	2.031	0.012	–0.898	0.254	15	–3.543	**0.003**
Ectomycorrhizal	*Tricholoma terreum*	2.207	0.033	–0.649	0.299	15	–2.17	**0.046**
Saprotrophic	*Lycoperdon perlatum*	0.715	0.695	0.068	0.212	15	0.32	0.753
Saprotrophic	*Leucopaxillus gentianeus*	13.853	2.411	–1.509	0.411	15	–3.67	**0.002**
Saprotrophic	*Clitocybe phaeophthalma*	0.022	0.000	–0.057	0.034	15	–1.689	0.112
Saprotrophic	*Hypholoma fasciculare*	10.706	2.018	–1.06	0.468	15	–2.28	**0.038**
Saprotrophic	*Gymnopus androsaceus*	0.029	0.015	–0.03	0.049	15	–0.604	0.555
Saprotrophic	*Mycena pura*	6.853	1.864	–1.107	0.23	15	–4.809	**<0.001**
Yeasts	*Cryptococcus* sp.	9.830	80.908	4.111	1.088	15	3.78	**0.002**
Yeasts	*Rhodotorula baccarum*	6.063	32.247	2.241	0.746	15	3.00	**0.009**
Yeasts	*Cryptococcus wieringae*	3.586	29.628	2.571	0.784	15	3.28	**0.005**
Yeasts	*Rhodotorula colostri*	0.389	1.348	0.502	0.146	15	3.435	**0.004**
Molds	*Mortierella elongata*	0.201	4.509	0.825	0.499	15	1.651	0.119
Molds	*Trichoderma* sp.	0.498	0.507	0.09	0.154	15	0.587	0.5658

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578 TABLE 3. Main sampling plot characteristics (altitude, location, and mean *Lactarius vinosus*
579 mushroom biomass for the period 2008–2014). Coordinates X (represented as E-W x) and Y
580 (represented as N-S y) are expressed as European Datum 1950 (ED50) datum.

Plot	Altitude (meters above sea level)	E-W x	N-S y	Mean *L. vinosus* mushroom yield (kg × ha^{-1})
301c	1010	4578428	336601	0.67
303c	903	4579373	335531	0.64
305c	744	4580525	335839	140.47
307c	796	4580200	335673	115.57
309c	852	4579709	335445	53.09
312c	633	4580848	333675	30.77
313c	609	4580755	336541	1.72
316c	644	4580113	336211	5.59

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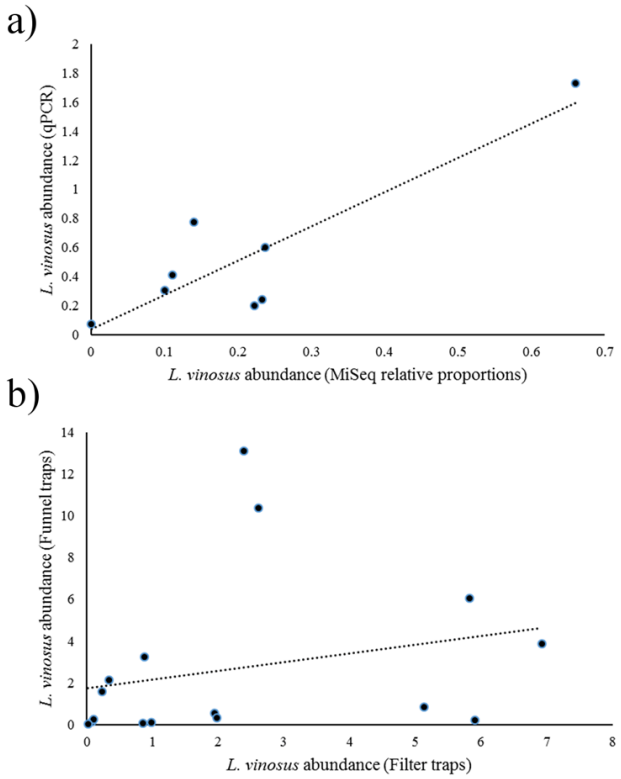
582

583 FIGURES

584



585

586 **FIG 1**: Relationship between the *L. vinosus* abundance measurements determined by qPCR
587 (y-axis) and by MiSeq data (x-axis) (a) and, relationship between the *L. vinosus* abundance
588 measurements obtained at the funnel traps (y-axis) and obtained at the filter traps (x-axis)

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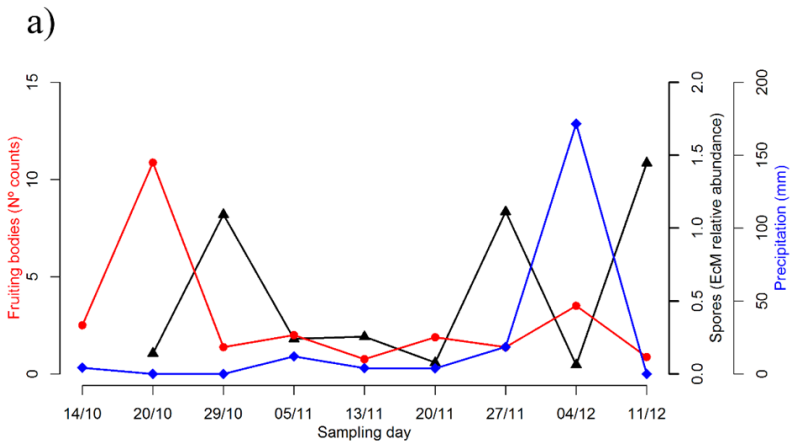

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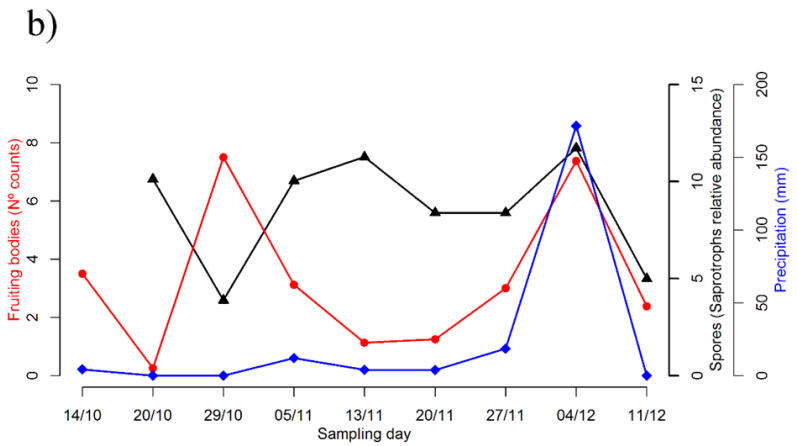
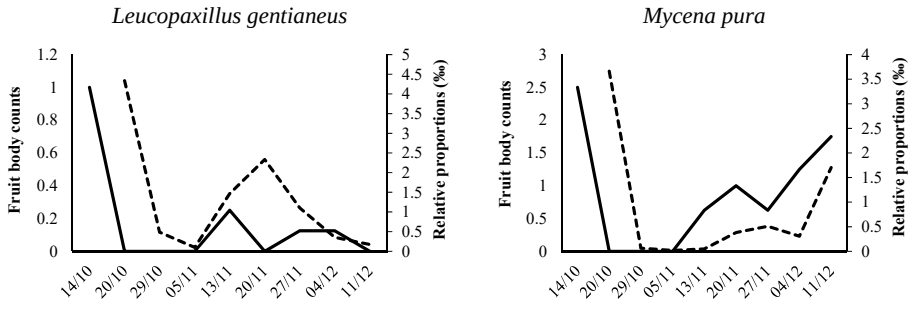


592 **FIG 2.** Temporal fluctuation of mushroom yields (red line) produced by (a) ectomycorrhizal
593 (EcM) and (b) saprotrophic species, the relative abundance of their spores (black line), and
594 the total precipitation recorded each week (blue line). The sampling day and month are shown
595 along the x-axis. During the first two weeks there were no precipitation events, and only low
596 levels of precipitation over the following weeks. However, during the first week of December,
597 an extreme precipitation event was recorded (almost 180 mm).

598



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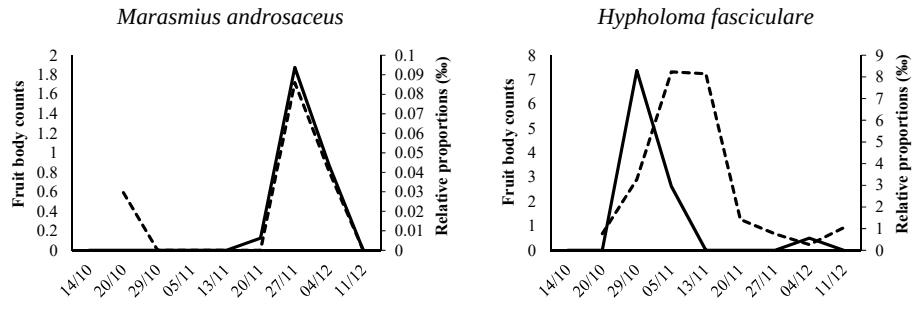

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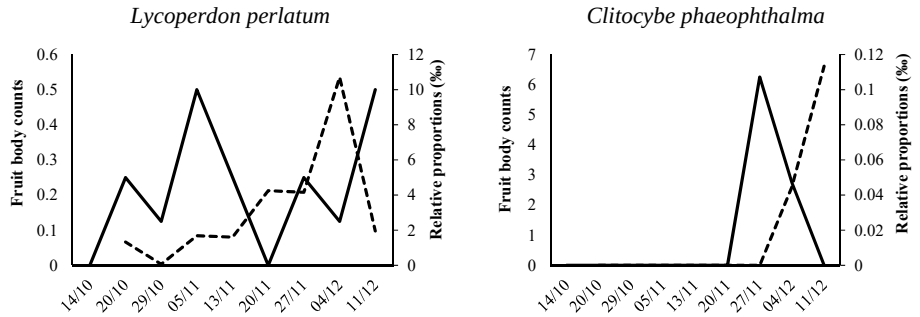

601 **FIG 3a.** Temporal changes in the number of fruiting bodies produced by saprotrophic species
602 and their spores across the sampled weeks in all the plots considered in this study. The solid
603 black line represents mushroom production (number of fruiting bodies × plot) and the broken
604 black line indicates the relative spore abundance (‰).

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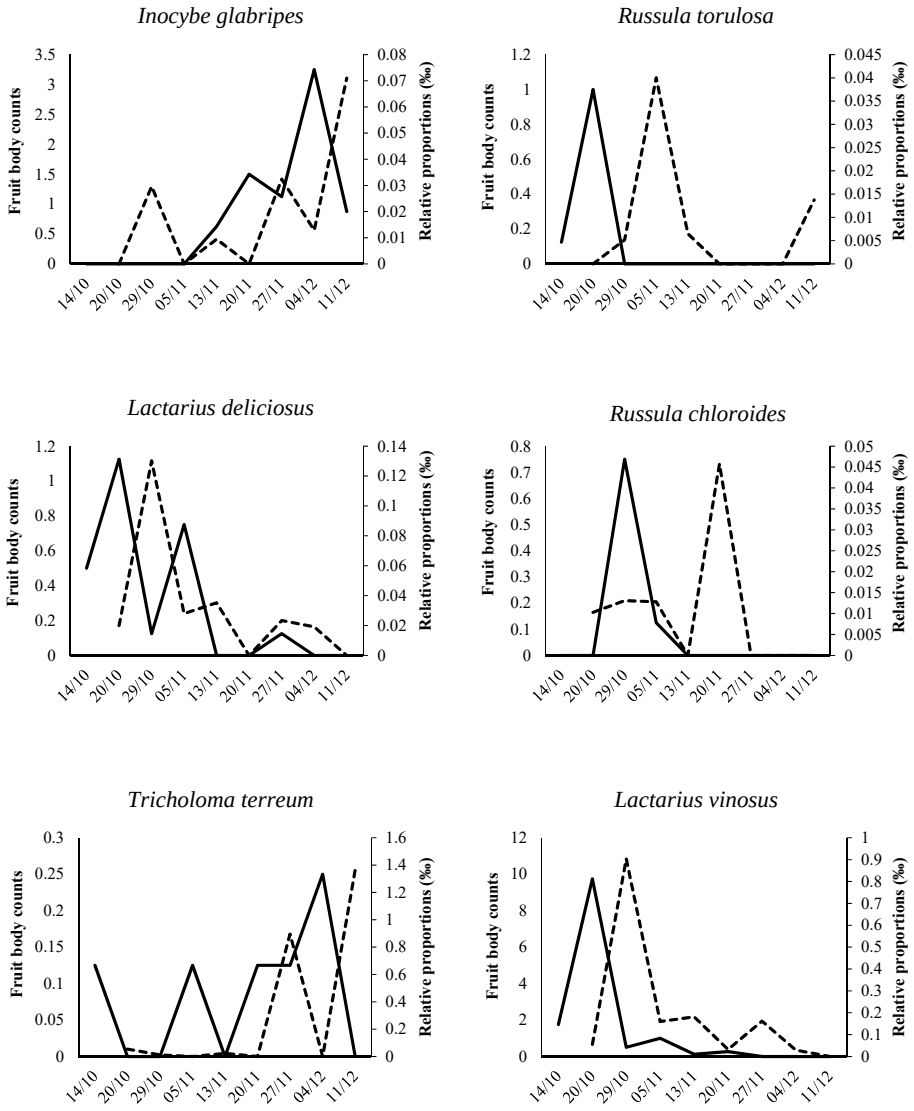
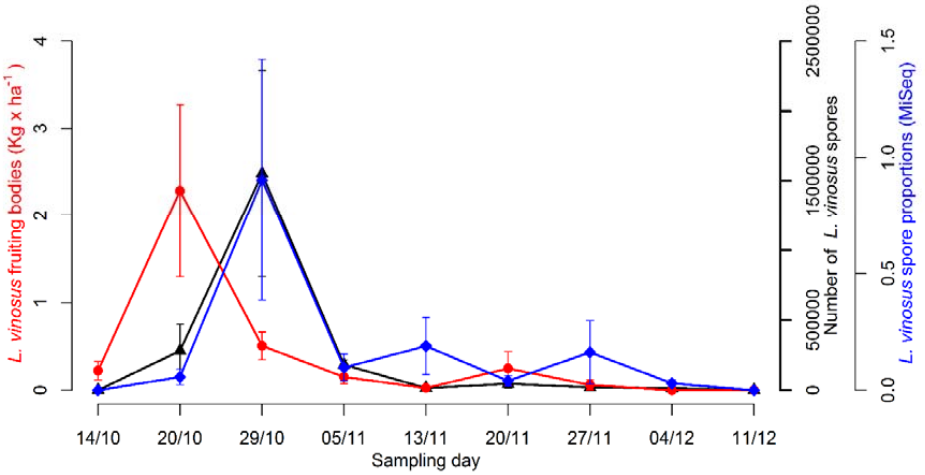


608 **FIG 3b.** Temporal changes in the number of fruiting bodies produced by ectomycorrhizal
609 species and their spores across the sampled weeks in all the plots considered in this study. The
610 solid black line represents mushroom production (number of fruiting bodies × plot) and the
611 broken black line indicates the relative spore abundance (‰).

612



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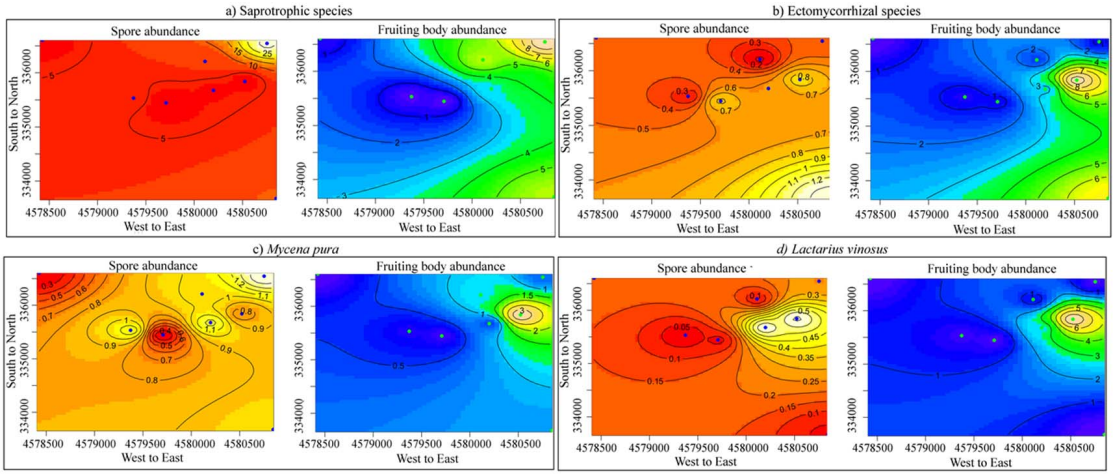
614 **FIG 4.** Relationship between mushroom biomass (red line) and the relative abundance of *L.*
615 *vinosus* spores detected by the spore traps, quantified by qPCR (black line) and by
616 determining the relative proportions of *L. vinosus* using MiSeq (blue line). Average values are
617 shown with their standard error. The sampling day and month are shown along the x-axis. The
618 peak in mushroom production on October 20th was detected the following week (October
619 29th) using both qPCR and MiSeq. The slight increase in mushroom production on November
620 20th was only detected by MiSeq.



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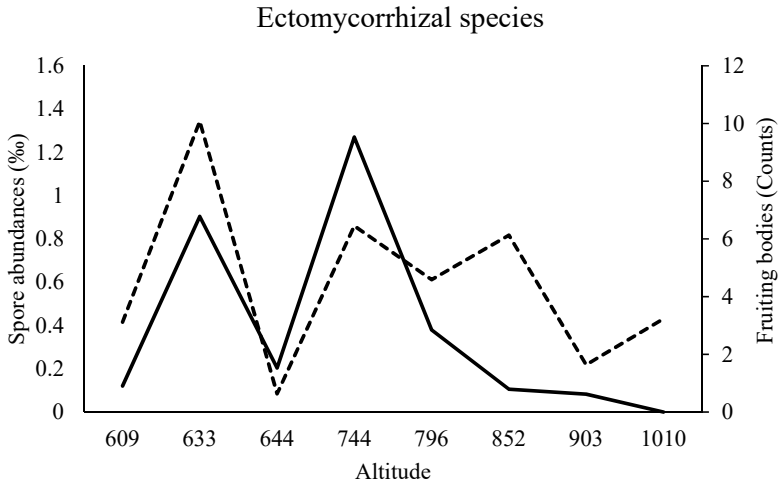
622 **FIG 5.** Spatial relationships between fruiting body yields (no. of fruiting bodies) and the relative abundance of spores evaluated by MiSeq
623 for (a) saprotrophic species, (b) ectomycorrhizal species, (c) *Mycena pura*, and (d) *Lactarius vinosus*. Blue or green dots represent each of
624 the 8 plots considered in this study. Reddish to yellowish colors in spore abundance represent a gradient from lower to higher spore
625 abundance, respectively, whereas blueish to orange colors represents a gradient from lower to higher fruiting body abundance, respectively.
626 Coordinates X (Easting) and Y (Northing) are represented in ED-50 datum.



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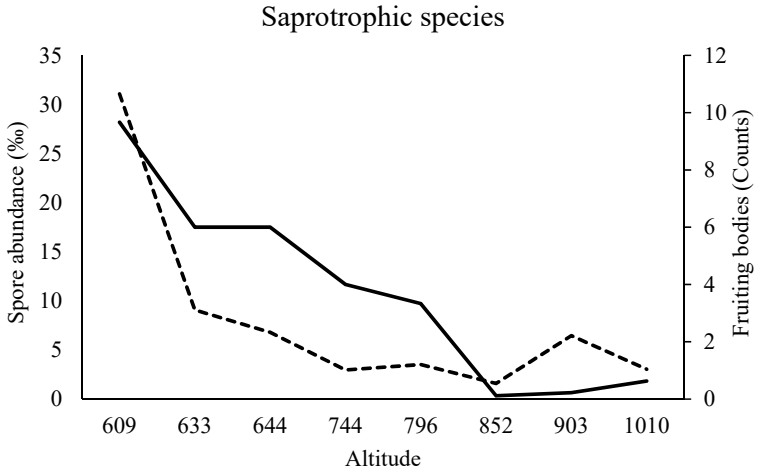

628

629 **FIG 6.** Relationship between fruiting body yields (solid line) and their propagules (broken
630 line) for (a) ectomycorrhizal and (b) saprotrophic species along an elevation gradient
631 represented by all the sampled plots considered in this study

TABLE 1. Selected most abundant species (species found at least twice in two different plots). Identification was based on comparison with ITS2 reference sequences deposited in UNITE. Ecology refers to the functional guild of the species (ectomycorrhizal or saprotrophic). Frequency refers to the order of frequency in terms of the total number of MiSeq reads among the whole propagule community.

Potential species	Ecology	Reference	Species Hypothesis Concept	DOI	Frequency
Inocybe glabripes Ricken	Ectomycorrhizal	UDB000099	*Inocybe glabripes*	DOI: 10.15156/BIO/SH174191.07FU	1620
Lactarius deliciosus (L.) S. F. Gray	Ectomycorrhizal	UDB011514	*Lactarius deliciosus*	DOI: 10.15156/BIO/SH220107.07FU	227
Lactarius vinosus Quél	Ectomycorrhizal	UDB000876	*Lactarius semisanguifluus*	DOI: 10.15156/BIO/SH220111.07FU	104
Russula chloroides (Kromb.) Bres.	Ectomycorrhizal	FM999633	*Russula chloroides*	DOI: 10.15156/BIO/SH220523.07FU	260
Tricholoma terreum (Sch.) Kumm.	Ectomycorrhizal	UDB015079	*Tricholoma gausapatum*	DOI: 10.15156/BIO/SH219345.07FU	168
Russula torulosa Bres.	Ectomycorrhizal	UDB011110	*Russula torulosa*	DOI: 10.15156/BIO/SH186211.07FU	877
Clitocybe phaeophthalma (Pers.) Kuyper	Saprotrophic	JX514120	*Singerocybe umbilicata*	DOI: 10.15156/BIO/SH191019.07FU	1709
Hypholoma fasciculare (Huds.) Kumm.	Saprotrophic	KF373785	*Hypholoma fasciculare*	DOI: 10.15156/BIO/SH201439.07FU	32
Leucopaxillus gentianeus (Quél.) Kotl.	Saprotrophic	UDB011628	*Leucopaxillus gentianeus*	DOI: 10.15156/BIO/SH030478.07FU	50
Marasmius androsaceus (L.) Fr.	Saprotrophic	AF519893	*Gymnopus androsaceus*	DOI: 10.15156/BIO/SH183805.07FU	1680
Mycena pura (Pers.) Kumm.	Saprotrophic	JF908417	*Mycena diosma*	DOI: 10.15156/BIO/SH186350.07FU	79
Lycoperdon perlatum Pers.	Saprotrophic	UDB023596	*Lycoperdon perlatum*	DOI: 10.15156/BIO/SH175885.07FU	51

TABLE 2: Differences in the relative proportions of ectomycorrhizal and saprotrophic species detected by filter traps vs. funnel traps. A few of the most abundant yeast and mold species are also included in the analysis to determine whether the relative proportion of ectomycorrhizal and saprotrophic species detected by funnel traps was smaller than that detected by filter traps owing to the trapping of a larger proportion of yeast or mold species by funnel traps.

		Mean proportions		Effects				
Ecology	Species	Filter trap	Funnel trap	Value	SE	df	*t*-value	*P*-value
Ectomycorrhizal	*Russula torulosa*	0.354	0.003	–0.262	0.126	15	–2.082	0.055
Ectomycorrhizal	*Inocybe glabripes*	0.079	0.015	–0.089	0.07	15	–1.267	0.225
Ectomycorrhizal	*Lactarius deliciosus*	2.408	0.075	–1.09	0.228	15	–4.793	**<0.001**
Ectomycorrhizal	*Lactarius vinosus* (MiSeq)	5.551	0.450	–1.531	0.307	15	–4.982	**<0.001**
	Lactarius vinosus (qPCR)	2.260	2.676	–0.016	0.268	15	–0.062	0.951
Ectomycorrhizal	*Russula chloroides*	2.031	0.012	–0.898	0.254	15	–3.543	**0.003**
Ectomycorrhizal	*Tricholoma terreum*	2.207	0.033	–0.649	0.299	15	–2.17	**0.046**
Saprotrophic	*Lycoperdon perlatum*	0.715	0.695	0.068	0.212	15	0.32	0.753
Saprotrophic	*Leucopaxillus gentianeus*	13.853	2.411	–1.509	0.411	15	–3.67	**0.002**
Saprotrophic	*Clitocybe phaeophthalma*	0.022	0.000	–0.057	0.034	15	–1.689	0.112
Saprotrophic	*Hypholoma fasciculare*	10.706	2.018	–1.06	0.468	15	–2.28	**0.038**
Saprotrophic	*Gymnopus androsaceus*	0.029	0.015	–0.03	0.049	15	–0.604	0.555
Saprotrophic	*Mycena pura*	6.853	1.864	–1.107	0.23	15	–4.809	**<0.001**
Yeasts	*Cryptococcus* sp.	9.830	80.908	4.111	1.088	15	3.78	**0.002**
Yeasts	*Rhodotorula baccarum*	6.063	32.247	2.241	0.746	15	3.00	**0.009**
Yeasts	*Cryptococcus wieringae*	3.586	29.628	2.571	0.784	15	3.28	**0.005**
Yeasts	*Rhodotorula colostri*	0.389	1.348	0.502	0.146	15	3.435	**0.004**
Molds	*Mortierella elongata*	0.201	4.509	0.825	0.499	15	1.651	0.119
Molds	*Trichoderma* sp.	0.498	0.507	0.09	0.154	15	0.587	0.5658

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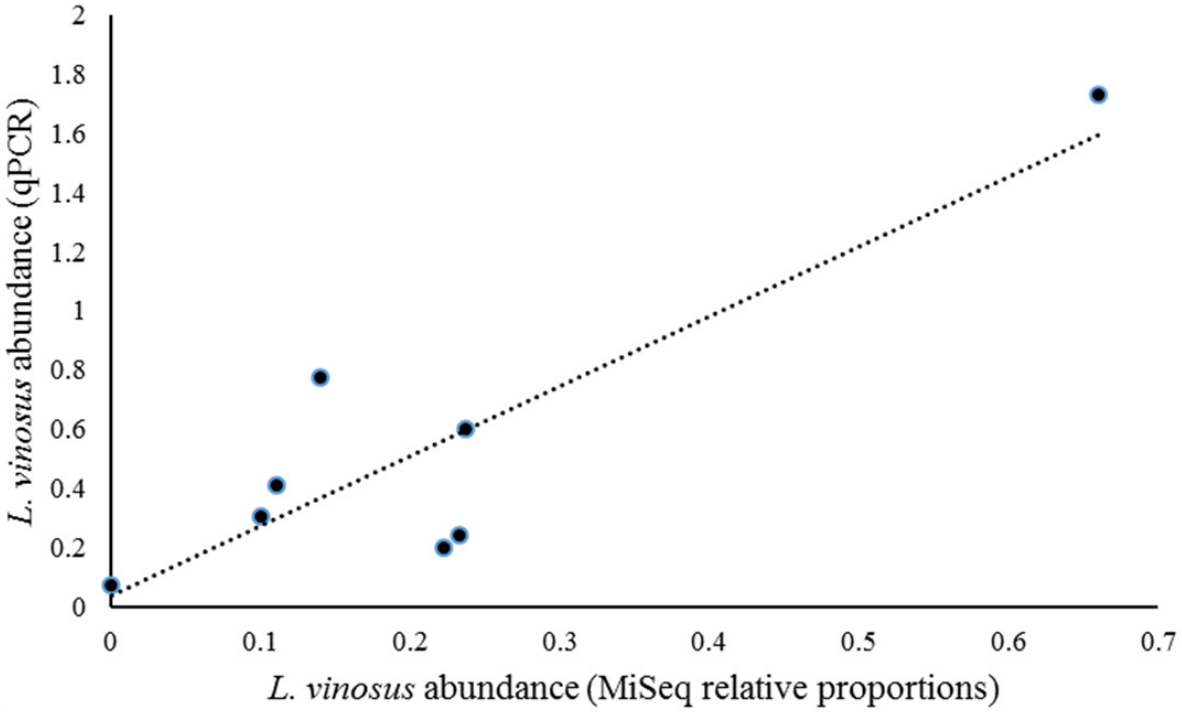
TABLE 3. Main sampling plot characteristics (altitude, location, and mean *Lactarius vinosus* mushroom biomass for the period 2008–2014). Coordinates X (represented as E-W x) and Y (represented as N-S y) are expressed as European Datum 1950 (ED50) datum.

Plot	Altitude (meters above sea level)	E-W x	N-S y	Mean *L. vinosus* mushroom yield (kg × ha^{-1})
301c	1010	4578428	336601	0.67
303c	903	4579373	335531	0.64
305c	744	4580525	335839	140.47
307c	796	4580200	335673	115.57
309c	852	4579709	335445	53.09
312c	633	4580848	333675	30.77
313c	609	4580755	336541	1.72
316c	644	4580113	336211	5.59

a)



b)